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Chromosome number, size and interspecific crossing ability of three Old World lupins, *Lupinus princei* Harms, *L. atlanticus* Gladstones and *L. digitatus* Forsk l, and implications for cyto-systematic relationships among the rough-seeded lupins

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Abstract

The chromosome number, size and interspecific crossing ability of several rough-seeded lupins were examined in a breeding programme for domestication of these species. The chromosome number of *Lupinus princei* was found to be n=19 (2n=38), while two other members of the rough-seeded lupin group, *L. atlanticus* and *L. digitatus*, were confirmed to have n=19 (2n=38) and n=18 (2n=36), respectively. *L. princei* had the largest chromosomes of any of the rough-seeded lupins, and failed to produce viable F1 seeds on crossing with *L. cosentinii* (n=16), *L. atlanticus*, *L. digitatus* and *L. pilosus* (n=21). In contrast, *L. digitatus* and *L. atlanticus* produced fertile interspecific hybrids between each other and with *L. cosentinii*. These three species had similar chromosome size. Previous reports indicate that *L. pilosus* and *L. palaestinus* (both n=21, 2n=42) form fertile hybrids and have the smallest chromosomes of the rough-seeded lupins. We confirm that *L. pilosus* does not form fertile F1's with *L. atlanticus*, *L. cosentinii* or *L. digitatus*. All the rough-seeded lupin species studied had high autofertility and pollen viability. Based on this information, we conclude that there are three discernible groups within the rough-seeded lupins: the Princei group (*L. princei*) in equatorial Africa, the Atlanticus group (*L. atlanticus*, *L. digitatus* and *L. cosentinii*) which overlap geographically in north-western Africa, and the Pilosus group (*L. pilosus* and *L. palaestinus*) in the eastern Mediterranean.

Introduction

When Gladstones (1974) reviewed the Old World lupins he resolved them into twelve species, distinguishing the morphologically similar "rough-seeded" lupins (lupins with rough seed testas) from the "smooth-seeded" lupins and describing a new rough-seeded lupin species from Morocco, *Lupinus atlanticus* Gladstones. The rough-seeded lupins have been separated in the genus at the rank of section, Sect. *Scabrispermae* Plitmann & Heyn (Plitmann and Heyn 1984). Systematic relationships within the rough-seeded lupins have been difficult to identify because they are morphologically uniform, and this is exemplified in Gladstones' (1974) description of *L. digitatus* Forsk.: "It has characters in common with *L. princei* and *L. somaliensis* to the south, and with *L. pilosus*, *L. atlanticus* and *L. cosentinii* to the north."

Genomic characters have potential for identifying systematic relationships at the infrageneric level, and the two most widely used characters are chromosome number and crossability. These characters have been used to establish a close taxonomic relationship between *L. pilosus* Murray and *L. palaestinus* Boiss which cross readily and share a chromosome number n=21 (Tuschnjakowa 1935, Kazimierski 1961, Pazy *et al.* 1977, Plitmann *et al.* 1980, Pazy *et al.* 1981). While the chromosome number of *L. cosentinii* Guss. (n=16) has been well established (Malheiros 1942, Gladstones 1958, Pazy *et al.* 1977), *L. princei* Harms has not been investigated and only unpublished reports exist of chromosome numbers for *L. atlanticus* (n=19) (Pazy, unpublished, cited in Plitmann *et al.* 1980) and *L. digitatus* (n=18) (Plitmann and Heyn, unpublished, cited in Plitmann and Heyn 1984). A report of n=18 also exists for *L. tassilicus* Maire (Eichorn 1949) which is believed to be synonymous with *L. digitatus* (Gladstones 1974). *L. somaliensis* Baker is known only from a single herbarium specimen and although suggested by Gladstones (1974) to be a distinct species, there have been no further records of its existence in Somalia since the original collection was made in the Golis Range before 1895 (M Thulin, Uppsala University, Sweden, personal communication).

An understanding of the interspecific relationships between the extant species in the rough-seeded lupins is essential in a breeding programme to domesticate this group. *L. atlanticus* and *L. pilosus* show potential as crops in Western Australia, especially on fine-textured neutral or alkaline soils (Roy and Gladstones 1983, Gladstones 1984, Buirchell and Cowling 1989). These two species may benefit from domestication genes transferred to them from *L. cosentinii*, which was the first (and as yet, only) rough-seeded lupin species to be fully domesticated (Gladstones 1982).

L. cosentinii was introduced into Western Australia more than one hundred years ago and it quickly became naturalized (Gladstones 1982). Known locally as the "Western Australian Blue Lupin" or "Sandplain Lupin", it occurs in coastal areas of south-west Western Australia. It is often sown into pastures for sheep feed and soil improvement where it regenerates for several years (Gladstones 1958).

In this paper we report the chromosome number of *L. princei*, and confirm unpublished chromosome number records for *L. atlanticus* and *L. digitatus*. The range in size of chromosomes is documented for these species, and the breeding systems of *L. princei*, *L. atlanticus* and *L. digitatus* are reported. Interspecific crossing data are then used to explore cyto-systematic relationships among the rough-seeded lupin group.

Materials and Methods

The *L. atlanticus* accession used in chromosome determinations, Commonwealth Plant Introduction (CPI) number 65223, originated from the Atlas Mountains of Morocco; the *L. digitatus* accession (CPI 120486) originated from Egypt; and the *L. princei* accession (CPI 120487) originated from Kitali, Kenya. Collection site details are described in Clements and Cowling (1990). Several other accessions were used in interspecific crossing experiments. Seeds are held in the Australian Lupin Collection, and small samples are available upon request from the authors.

Meiosis was examined in pollen mother cells of young anthers from five plants for each species. Bud material was fixed in ethanol:acetic acid (3:1) at 50˚C for 1 hr. The buds were transferred to 70% ethanol at 50˚C for 1 hr, then bulk stained in alcoholic hydrochloric acid carmine (Snow 1963) for 5-14 days before analysis.

Pollen viability was determined by calculating the frequency of pollen grains that were stained with lactophenol-cotton blue. Autofertility was determined by calculating the percentage of ovules that developed into seeds in a sample of pods from plants that were isolated from insect pollinating vectors in insect-proof glasshouses or screenhouses.

Mitotic metaphases were examined in dividing root tip cells. Seeds were scarified and germinated on blotting paper in petri dishes at 22-27˚C for 3-4 days. Root tips were cold treated with or without 0.1% colchicine at 2-3.5˚C for 2.5-3.5 hr, then fixed in ethanol:acetic acid (3:1) for 5 min at 50˚C, transfered to new fixative and kept at 3.5˚C. After 18-48 hr the root tips were hydrolyzed in 0.25 N HCl at 45-56˚C for 1.5-2.5 min, then squashed in lacto-aceto orcein (Darlington and La Cour 1962). Metaphase chromosome lengths were determined from photographic enlargements with Vernier callipers.

A number of interspecific crosses were attempted with four different accessions of *L. princei*. Pollen from several accessions of *L. atlanticus* and *L. pilosus* were used along with one accession each of *L. digitatus* and *L. palaestinus* and three accessions of *L. cosentinii*. *L. princei* was emasculated 24 hr prior to pollination when used as the female plant. Reciprocal crosses were also made. Several hundred interspecific crosses were attempted between at least fifteen accessions each of *L. pilosus* and *L. atlanticus*, and a smaller number between *L. pilosus* and *L. cosentinii* or *L. digitatus*.

Results and Observations

Chromosome numbers

We report chromosome counts for three rough-seeded lupin species as follows:

Lupinus princei Harms: n=19 and 2n=38 (Figs 1A and 1B). No previous records exist.

Lupinus atlanticus Gladstones: n=19 and 1n=38 (Figs 1C and 1D). Our determination confirms the only other previous unpublished record of 2n=38 (Pazy, unpublished, cited in Plitmann *et al.* 1980), and establishes that *L. atlanticus* and *L. princei* have the chromosome number n=19 in common.

Lupinus digitatus Forsk l: n=18 and 2n=36 (Figs 1E and 1F). Our determination confirms two previous counts of 2n=36 (Eichorn 1949 for the synonymous species *L. tassilicus*; and Plitmann & Heyn unpublished, cited in Plitmann & Heyn 1984).

Pollen mother cell meiosis

During metaphase I of meiosis, 19 bivalents were regularly observed in all the *L. princei* and *L. atlanticus* plants examined (Figs 1A and 1C respectively). Eighteen bivalents were observed at metaphase I in all the *L. digitatus* plants examined (fifty five cells from four plants). No multivalents were observed in any of the cells of the species examined. Anaphase I was regular in *L. digitatus* (Fig 1E) and the other two species (forty cells from five plants of each species). Subsequent meiotic stages were also regular in all three species.

Pollen viability and reproductive biology

The observed meiotic regularity was confirmed in pollen viability tests. Pollen stainability for all three species approached 100% (Table 1), which is similar to that determined by Pazy *et al.* (1977) for *L. palaestinus* (96%) and *L. pilosus* (93.6%). We observed that *L. princei*, *L. atlanticus* and *L. digitatus* were all autofertile annual herbs, and set a high frequency of fully developed seed when isolated from insect pollinating vectors (Table 1). It is likely that autofertility, which has also been reported for *L. cosentinii* (Gladstones 1974), *L. pilosus* and *L. palaestinus* (Plitmann *et al.* 1980), accounts for the majority of seeds set in nature by the species we studied.

Root-tip mitosis chromosome sizes and complement symmetry

The lupin species examined in this study had relatively small chromosomes (Table 1) compared with other angiosperms where chromosome lengths may exceed 14 µm (Levin & Funderberg 1979). The chromosomes of *L. princei* are the largest of any lupin species studied to date (compared with Gilot 1965, Pazy *et al.* 1977), and were 1.5-1.65 times the size of *L. atlanticus* and *L. digitatus* chromosomes (Table 1). Pazy *et al.* (1977) determined *L. cosentinii* chromosomes to be about 3 µm in length, which is comparable with the size of *L. atlanticus* and *L. digitatus* chromosomes (Table 1).

The centromeres were in the median and sub-median regions of the mitotic metaphase chromosomes in all the species that were studied. Prominent secondary constrictions (nucleolar organizer regions) and trabants were a feature of a large pair of homologous chromosomes in each species (Figs 1B, 1D, 1F). The chromosome compliments were asymmetrical, and *L. digitatus* had the narrowest range of chromosome lengths (Table 1).

Geographic distribution of chromosome numbers in the rough-seeded lupins

Results from this study and previous reports indicate that chromosome numbers of the rough-seeded lupins are not distributed according to any geographical pattern, and no particular chromosome number is most common for the group (Table 2). *L. atlanticus* and *L. princei* are the two most disjunct species (Fig 2), occurring 5,000 km apart, and share a common chromosome number (n=19). This is in contrast with *L. pilosus* and *L. palaestinus* which also share a common chromosome number (n=21) and whose distributions are contiguous or parapatric. *L. digitatus* is documented to be the most widely distributed rough-seeded lupin species (Gladstones 1974), and it has an intermediate chromosome number for the group.

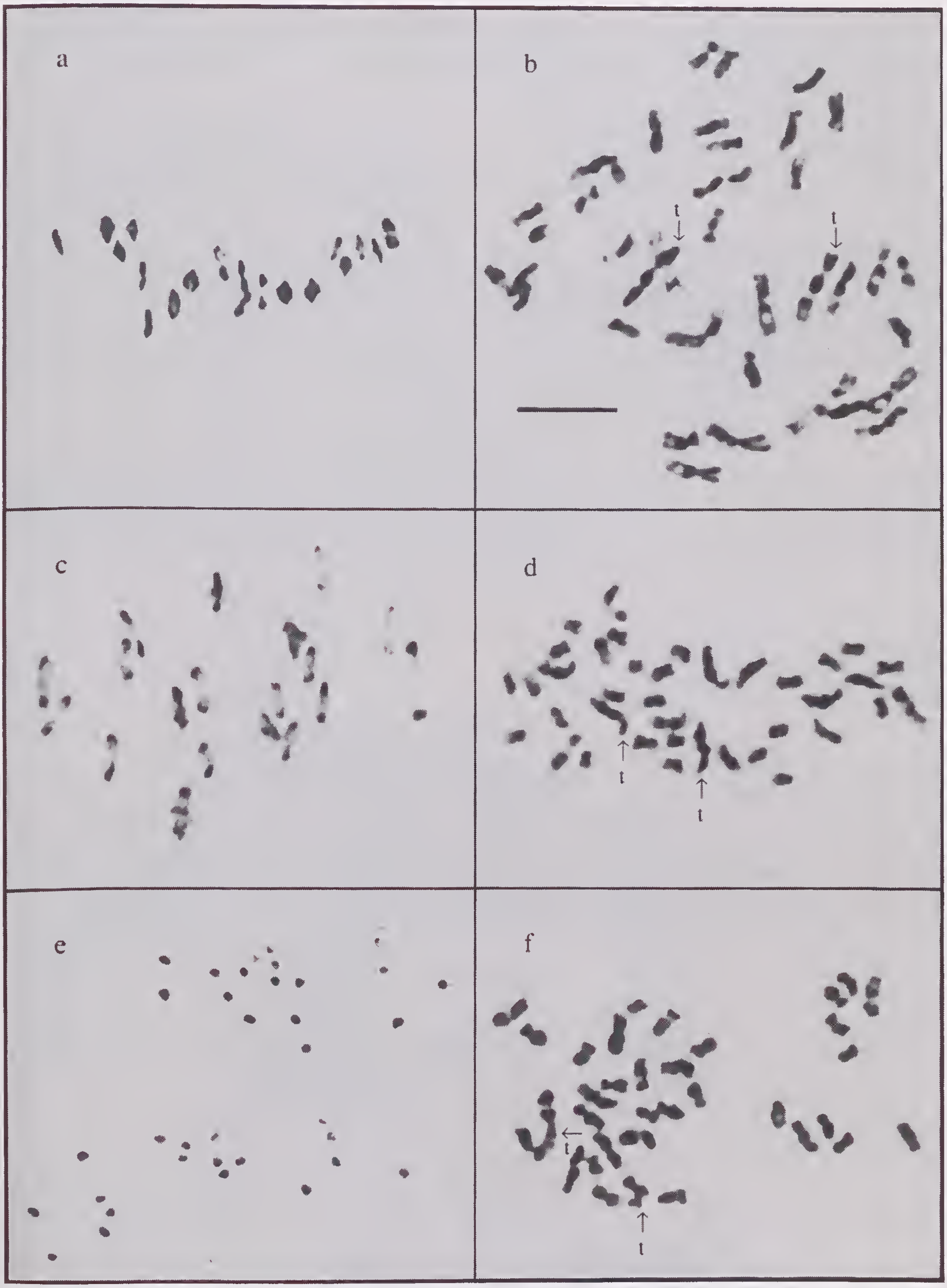

Figure 1. Pollen mother cell meiosis and root-tip mitosis in: A. *L. princei*: metaphase I of meiosis, n = 19. B. *L. princei*: metaphase of mitosis, 2n = 38. C. *L. atlanticus*: metaphase I of meiosis, n = 19. D. *L. atlanticus*: metaphase of mitosis, 2n = 38. E. *L. digitatus*: anaphase I of meiosis, n = 18. F. *L. digitatus*: metaphase of mitosis, 2n = 36. Bar represents 5.5 μm (applies to B, D and F only). Trabants are indicated with the letter 't'.

Crossability of L. princei *with other species*

It was anticipated that *L. cosentinii, L. digitatus* and *L. atlanticus*, which produce viable F1 seeds when crossed to each other (Roy and Gladstones 1985, 1988), would cross to *L. princei* because of the similarity or closeness of their chromosome numbers. However, the results proved otherwise. All interspecific crosses using *L. princei* as the pollen receptor failed to develop fertile F1 seed. Although pod development did proceed, the seeds were shrivelled and lacked an embryo. The reciprocal crosses were also unsuccessful.

Crossability of L. pilosus *with other lupin species*

Interspecific crosses of *L. pilosus* with *L. atlanticus, L. digitatus* and *L. cosentinii* failed to produce fertile F1 plants confirming previous reports of Roy and Gladstones (1988). F1 plants of *L. pilosus/L. atlanticus* or *L. pilosus/L. cosentinii* grew vigorously and flowered profusely but all failed to produce viable seeds. Two F2 seeds, intermediate in size between the parent seed types, were obtained from one cross of *L. pilosus/L. atlanticus* but these failed to germinate normally and died after radicle emergence and before roots developed.

Discussion

The common chromosome number of *L. princei* and *L. atlanticus* (n=19) has cytogeographic significance for the rough-seeded lupins. Two species sharing the same chromosome number is not unique for the group as *L. pilosus* and *L. palaestinus* both have n=21, but the two cases are different. In contrast to *L. pilosus* and *L. palaestinus*, whose respective geographical distributions overlap, *L. princei* and *L. atlanticus* are geographically separated on opposite sides of the African continent, and occur in different climatic zones. *L. princei* is found in the equatorial highlands of tropical E and NE Africa (Kenya, Tanzania and southern Ethiopia), while *L. atlanticus* grows in the foothills

Table 1

Range of mitotic metaphase chromosome lengths, pollen fertility and autofertility for *L. princei, L. atlanticus* and *L. digitatus*

Species	Range of chromosome lengths (µm)	Pollen viability (%)			Autofertility			
		Mean	Range	No. plants examined	No. ovules	No. pods	No. seed	Fertile ovules (%)
L. princei	1.77-4.40	98.6	97-99	5	96	26	89	92.7
L. atlanticus	0.97-3.02	98.0	96-99	3	78	22	76	97.4
L. digitatus	1.16-2.91	92.4	73-98	11	200	50	175	87.5

Table 2

Previous chromosome number determinations of the rough-seeded lupins and geographical distributions of the species

Species	Chromosome number	Source	Distribution
L. pilosus Murray	2n = 42	Tuschnjakowa 1935 cf. Gladstones 1958	E. Mediterranean coastal to 1,200 m Greece, Turkey, Syria, Israel
	n = 21	Pazy *et al.* 1977	
L. palaestinus Boiss	2n = 42 n = 21	Pazy *et al.* 1977	Central and S Israel, Sinai Peninsula—semi-arid to desert regions
L. atlanticus Gladstones	2n = 38	Pazy (unpublished) cited in Plitmann *et al.* 1980	Morocco, Anti Atlas & foothills of High Atlas mountains—600-1,500 m
	2n = 38 n = 19	This study	
L. digitatus Forsk 1	2n = 36	Eichorn 1949 (as *L. tassilicus* Maire)	N. Africa, Egypt, Sahara—few confirmed records (Gladstones 1974)
	2n = 36	Cited in Plitmann & Heyn 1984 (unpublished)	
	2n = 36 n = 18	This study	
L. cosentinii Guss	2n = 32	Malheiros 1942	Coastal areas of W. Mediterranean—low altitude; Morocco, Spain and Tunisia
	2n = 32	Gladstones 1958	
	2n = 32	Pazy *et al.* 1977	
L. princei Harms	2n = 38 n = 19 (No prior determinations recorded)	This study	Equatorial, highlands of Kenya, Tanzania and S. Ethiopia—1,700-3,000 m
L. somaliensis Baker*	(No determinations)		Golis Range in NW Somalia

* *L. somaliensis* was described in 1895 from only one botanical specimen without seeds or pods (Gladstones 1974). The species, if genuine, is now believed to be extinct (M. Thulin, Uppsala University, Sweden, personal communication).

of the Anti Atlas and Atlas Mountains of Morocco where a Mediterranean type climate predominates (Gladstones 1974).

The size of *L. princei* chromosomes, the largest of all the rough-seeded lupins, also sets it apart from *L. atlanticus* and *L. digitatus* (Table 1). Chromosome size variation is potentially a useful taxonomic character (Rees and Jones 1977, Grielhuber 1977, Bennett 1984, Jones 1984) and genomic divergence occurs when the difference in DNA content is large enough to prevent successful crossing between species (Jones 1984).

Finally, *L. princei* failed to produce viable seed when crossed with any of the other rough-seeded lupin species. We conclude, therefore, that the genome of this species has diverged so much from the others that it has been rendered systematically isolated from the rest of the group.

Interspecific crossing between *L. atlanticus* (n=19), *L. digitatus* (n=18), and fully domesticated *L. cosentinii* cv Erregulla (n=16) resulted in fertile F1 progeny in all combinations (Roy and Gladstones 1985, 1988), indicating that they were closely related species. Domestication genes such as soft seeds and early flowering (non-vernalization requirement) were transferred from cv Erregulla to fertile hybrids of *L. atlanticus*/*L. cosentinii*, and from *L. digitatus* to a fertile hybrid of *L. atlanticus*/*L. digitatus*, respectively (B J Buirchell unpublished). A comparison of chromosome size, which is about the same for these three species (Table 1), also supports the close relationships among these species.

L. pilosus produces viable F1 progeny when crossed with *L. palaestinus* (both n=21) (Kazimierski 1961, Pazy *et al.* 1977). These two species have the smallest chromosomes of the rough-seeded lupin group, and their geographic range overlaps. Crossability data also indicates that they are distantly related to the other rough-seeded lupins. *L. pilosus* forms sterile F1 plants in crosses with *L. atlanticus* and *L. cosentinii* (Roy and Gladstones 1988, confirmed in this study). *L. princei* failed to produce viable F1 seeds in crosses with *L. pilosus*.

We therefore recognize three subgroups of the rough-seeded lupins based on chromosome number, size and interspecific crossing ability presented here and elsewhere: the Pilosus group with *L. pilosus* and *L. palaestinus*; the Atlanticus group with *L. atlanticus*, *L. digitatus* and *L. cosentinii*; and the Princei group with *L. princei* only. These groupings follow the natural geographic separation of the rough-seeded lupins: the Pilosus group occurs in the east Mediterranean region, the Atlanticus group converges in the vicinity of Morocco, and the Princei group is isolated in eastern equatorial Africa (Fig 2). We tentatively place *L. somaliensis* in the latter group awaiting confirmation of existence of this species in eastern Africa.

We have shown in this study that the rough-seeded lupins are cytologically diverse. They share this feature with the Old World smooth-seeded lupins, whose chromosome numbers are n=20, 25 and 26 from 5 species (Gladstones 1974, Pazy *et al.* 1977). The New World lupins, of which there are over 200 species, contrast in this respect

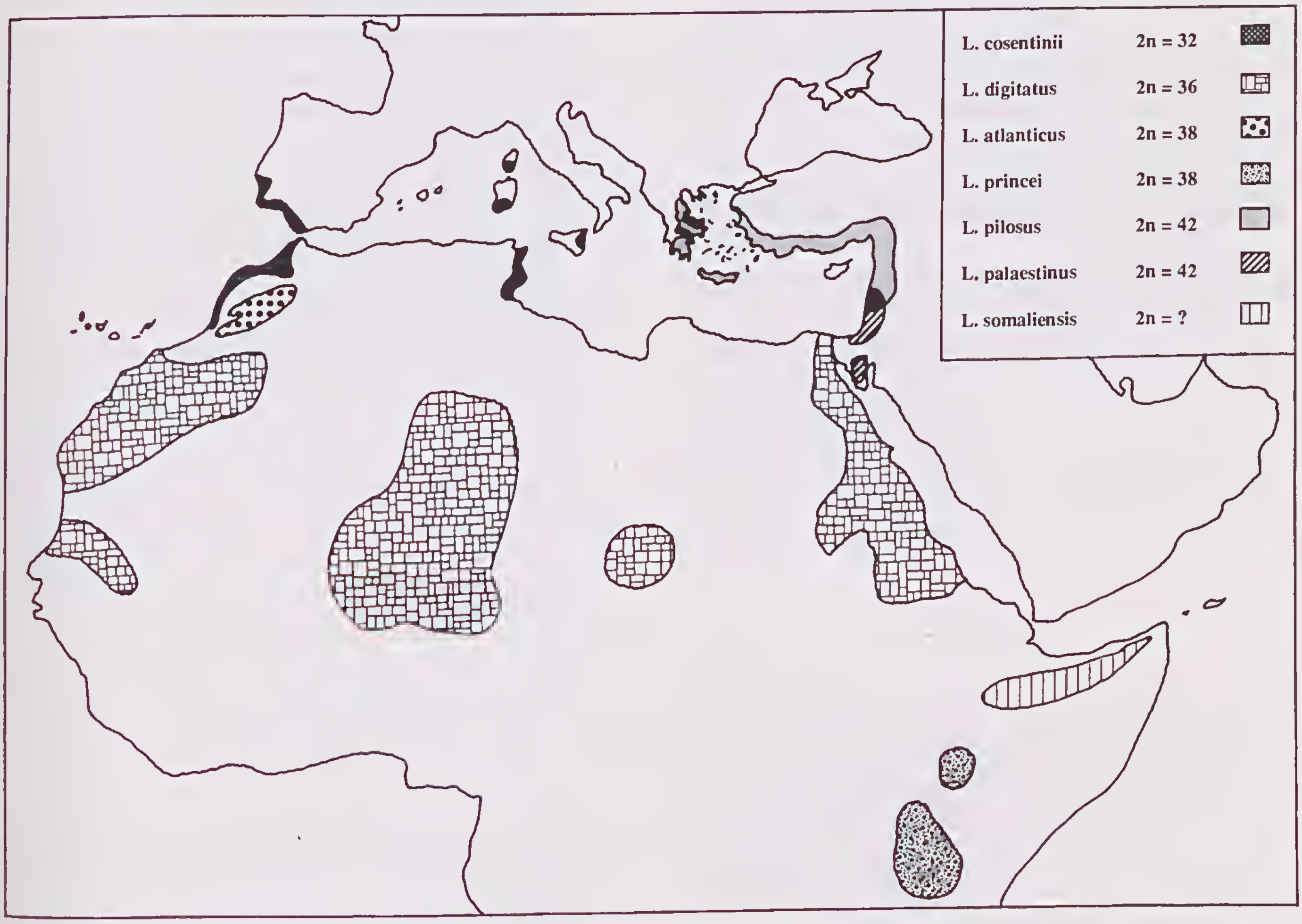

Figure 2. Distribution of the rough-seeded Old World lupins (from Gladstones 1974), with the chromosome numbers of their respective taxa.

with the Old World lupins. The New World lupins are cytologically uniform and characterized by n=24 (Dunn 1984), as well as being typically out-crossing species. This suggests that evolutionary forces operating in the New World have been different from those operating in the Old World. The climate in northern Africa has become drier since the Miocene. As suitable habitat dwindled, the rough-seeded lupins may have became geographically isolated from one another due to their restriction to wetter coastal areas, hills or oases. A consequence of this geographical isolation, reinforced by high autofertility and low gene flow, has been the evolution and conservation of a variety of genomic arrangements in this group, which is in contrast to their morphological uniformity.

Confirmation of the proposed relationships among the rough-seeded lupins will require more detailed karyotype analyses, observation of chromosome pairing in F1 progeny of interspecific crosses, and further interspecific crossing work. Some of this work is currently underway as part of a domestication programme for *L. atlanticus* and other rough-seeded lupins (Buirchell & Cowling 1989).

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